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(54) Title: RECEPTOR SPECIFIC CHIMERIC VIRAL SURFACE POLYPEPTIDES FOR VIRAL AND PARTICLE INCORPORATION AND INTERNALIZATION IN TARGET CELLS		
(57) Abstract <p>Viral and non-viral particles can be incorporated by chimeric viral surface polypeptides that are constructed to include a portion of a viral surface polypeptide and a portion of a ligand specific for a receptor of the LDL-receptor family. Particles incorporated by the chimera can target cells specific for the receptor type for which the chimera is designed, and can be internalized in these cells. Exemplary molecules of the invention include chimeras constructed with a ligand portion of a SERPIN, for example PAI-1, for targeting cells expressing unbound uPA, and for forming an internalizable complex upon contact with LRP cell surface receptor. Accordingly, when the particle is internalized in the target cell, so is any genome or polynucleotide encoding a polypeptide for expression that is contained in the viral or non-viral particle. Cell specific gene therapy protocols are thereby accomplished by the invention.</p>		

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RECEPTOR SPECIFIC CHIMERIC VIRAL SURFACE POLYPEPTIDES FOR VIRAL AND PARTICLE INCORPORATION AND INTERNALIZATION IN TARGET CELLS

Field of the Invention

The invention relates to methods of delivery of genes, proteins, ribozymes, antisense oligonucleotides, small molecules, and other therapeutic agents to target cells. Specifically, the invention relates to methods of internalization in target cells of viruses or particles containing molecular contents that include coding sequences for expression in a cell, and proteins, ribozymes, anti-sense oligonucleotides, small molecules, and other therapeutic agents for initiating, attenuating or inhibiting various molecular and cellular responses. The invention also relates to chimeric viral surface polypeptides for incorporation into viruses or particles, and to particles altered by the addition of a ligand portion of a receptor-specific ligand for internalizing the altered particle and its molecular contents in a target cell. Claimed herein are chimeric viral surface polypeptides, the polynucleotide sequences encoding the chimeras, a virus or particle incorporated with the chimeras, the virus or particles internalized by a target cell, altered particles possessing an internalizable ligand portion on their surface, and methods of treating diseases using the method of the invention for targeting viruses or particles to target cells in a cell specific manner.

Background of the Invention

The advent of gene therapy has brought the promise of expressing non-expressed genes in an animal. Gene therapy can be accomplished by viral or non-viral delivery of coding regions. Viral delivery of heterologous genes has been accomplished with some success using adenoviral vectors as described in Connelly *et al Human Gene Therapy* 6:185-193 (1995), adeno-associated viral vectors as described in Kaplitt *et al Nature Genetics* 8:148-211 (1994), and retroviral delivery as described in Kimura *et al Human Gene Therapy* 5:845-852 (1994). Other retroviral vectors and recombinant retroviruses are

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also described in PCT applications PCT/US 89/01139 and PCT/US 90/04652. Tissue specific viral delivery has also been achieved with some success using retroviral vectors and a chimeric protein containing the polypeptide hormone erythropoietin and part of the *env* protein of ectotropic Moloney murine leukemia virus engineered into the virus, as
5 described in Kasahara *et al Science* 266:1373-1376 (1994) and PCT application 93/25234 to Y.W. Kan.

Non-viral delivery has been accomplished by the injection of naked DNA plasmid containing the coding sequence for the heterologous protein as described in Zhu
et al Science 261:209-211 (1993). Non-viral delivery has also been accomplished by
10 inserting a coding sequence into conventional vectors that contain conventional control sequences for high level expression, and then incubating the construct with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu and Wu, *J. Biol. Chem.* (1987) 262: 4429-4432; insulin, as described
15 in Hucked *et al., Biochem. Pharmacol.* 40: 253-263 (1990); galactose, as described in Plank *et al., Bioconjugate Chem.* 3:533-539 (1992); lactose, as described in Midoux *et al., Nucleic Acids Res.* 21: 871-878 (1993); or transferrin, as described in Wagner *et al., Proc. Natl. Acad. Sci. USA* 87:3410-3414 (1990).

Other delivery systems include the use of liposomes to encapsulate DNA
20 comprising the gene under the control of a variety of tissue-specific or ubiquitously-active promoters, as described in Nabel *et al., Proc. Natl. Acad. Sci. USA* 90: 11307-11311 (1993), and Philip *et al., Mol. Cell Biol.* 14: 2411-2418 (1994). Liposomes are also appropriate for the delivery of other therapeutic agents including proteins and non-protein therapeutic agents. Further non-viral delivery suitable for use includes
25 mechanical delivery systems such as the biolistic approach, as described in Woffendin *et al., Proc. Natl. Acad. Sci. USA* (1994) 91(24): 11581-11585. Moreover, the coding sequence and the product of expression of such and other therapeutic agents such as, for example, those described herein, can be delivered through deposition of photopolymerized hydrogel materials. Other conventional methods for gene delivery

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that can be used for delivery of the coding sequence include, for example, use of hand held gene transfer particle gun, as described in U.S. 5,149,655; use of ionizing radiation for activating transferred genes, as described in U.S. 5,206,152 and PCT application WO 92/11033.

5 The limitations presently existing in the art of both viral and non-viral delivery include limited success accomplishing sustained, high level tissue specific incorporation of viral or other particles. Problems with non-specific viral and particle internalization occur in gene therapy when the expression of the coding sequence is desired in one cell type, but the virus infects many cell types, or in delivery systems generally, when it is
10 desired that a certain cell population internalize and use a delivered therapeutic, but when the therapeutic is instead internalized in cells in a non-specific manner, reducing the therapeutic dose to the target cell population.

It is desirable therefore, to devise a method of increasing the specificity of internalization of viruses or other particles in target cells.

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Summary of the Invention

It is an object of the present invention to provide a chimeric polynucleotide encoding a chimera of a ligand and a viral surface protein for use in delivery of a virus or a particle containing molecular contents for internalization into target cells such that the
20 chimera includes a polynucleotide sequence encoding all or a portion of a ligand of a receptor from the LDL-receptor family, and a polynucleotide sequence encoding all or a portion of a viral surface protein, so that the expression product of the chimeric polynucleotide forms a chimeric viral surface polypeptide capable of forming an incorporation aggregate for incorporation of a virus or particle, and capable of forming a
25 binding pair with the receptor for internalization of the virus or particle incorporated by the chimeric viral surface polypeptide into a target cell expressing the receptor, and wherein the virus or particle comprises molecular contents for delivery to a target cell.

It is a further object of the invention to provide a chimeric polypeptide for incorporation of a virus or particle for targeting a cell for internalization of the virus or

particle for delivery of viral or particle contents that includes a polypeptide sequence including all or a portion of a ligand of a receptor from the LDL-receptor family, and that also includes an additional polypeptide sequence including all or a portion of a viral surface protein, so that the chimeric polypeptide can make up an incorporation aggregate of these chimeric polypeptides for incorporation of a virus or particle to form an incorporated virus or incorporated particle for targeting a cell expressing the receptor. The targeting of a cell expressing the receptor occurs when the ligand forms a binding pair with the receptor for internalization of the incorporated virus or incorporated particle. The virus or particle can contain molecular contents for delivery to the target cell, including but not limited to genes for expression in the target cell, and therapeutics of all kinds for delivery to the target cells.

It is another object of the invention to provide a method of targeting and internalizing a virus or particle comprising the steps of (a) constructing a chimeric polynucleotide encoding a chimeric polypeptide that includes a polypeptide sequence including all or a portion of a ligand of a receptor from the LDL-receptor family, and that also includes an additional polypeptide sequence including all or a portion of a viral surface protein, (b) expressing the chimeric polynucleotide to form a chimeric viral surface polypeptide (c) incorporating the chimeric viral surface polypeptide into the surface of a virus or particle containing molecular contents to form an incorporated virus or particle, (d) bringing the incorporated virus or particle in contact with a target cell expressing the receptor (e) internalizing the virus or particle into the target cell by binding of the ligand with the receptor.

It is an additional object of the invention to provide an altered particle designed for delivery to target cells for internalization of molecular contents contained in the particle including a particle that contains molecular contents, a polypeptide moiety made up of all or a portion of a ligand of a receptor from the LDL-receptor family, designed in such a way that the particle and the ligand portion are connected to one another to form an altered particle for delivery of the molecular contents of the particle to a target cell.

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A further object of the invention is to provide for ligand portions of the chimeric viral surface polypeptides of the invention that include SERPINs (serine protease inhibitors), particularly PAI-1, and also including TFPI.

Further objects, features, and advantages of the present invention will become
5 apparent from the following detailed description. It should be understood, however, that the detailed description, while indicating preferred embodiments of the present invention, is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. The invention is also not limited to any theories of
10 action of the elements of the invention.

Detailed Description of the Invention

The present invention conceives a method of delivery of genes or other therapeutics using the internalization mechanism of viral surface proteins, cell surface
15 receptors, and the ligands for these receptors.

In the past, in the typical cases involving chimeric polypeptides for gene delivery, a ligand is expressed on a virus or other gene delivery particle and this ligand binds to and is internalized by a cell expressing the receptor. The specificity of this event is dependent on the amount and distribution of the receptor on the various cell
20 types, including the target cell. In this case if the receptor already has the ligand bound to it, for example erythropoietin bound to the EPO receptor, then a gene delivery particle can not bind to that cell, as described in PCT application 93/25234.

The present invention conceives of a chimeric viral surface protein that includes a ligand or a portion of a ligand specific for a receptor of the LDL-receptor family. Use
25 of these ligands surmounts the obstacle of reduced specificity where the receptors are bound to ligands and thereby unable to bind other ligands. In the present invention, the gene delivery particle is decorated with a ligand, which recognizes another ligand:receptor complex. In particular, for example, a gene delivery particle expressing

PAI-1 ligand, will recognize and to bind covalently to active two chain uPA bound to the uPAR receptor.

According to this example of the invention, only cells which are actively involved in cell surface plasminogen activation, for example, and are displaying active
5 receptor bound uPA, will be targeted by the gene delivery particle. After formation of the particle:uPA:uPAR complex, mediated by the binding of PAI-1 at the particle surface to uPAR bound uPA, efficient internalization of the particle will occur via transfer to LRP or related scavenger receptors. By the method of the invention enhanced specificity for target cells is achieved.

10 The invention includes all ligands of the LDL-receptor family, and thus can achieve specificities of target cell internalization on the basis of the cell types that express the receptors of the LDL-receptor family, and also on the basis of cell types that express other receptors that bind other ligands that bind the ligands of the LDL-receptor family, and later, as a ligand complex of one or more polypeptides, and still connected
15 to the original particle to be delivered to a cell, binds the receptor of the LDL-receptor family and is internalized.

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All published work, including patents, and patent
20 applications cited herein are hereby incorporated by reference.

The invention can be better understood in light of the following definitions incorporated herein.

Definitions

A "polynucleotide" or a "coding sequence," as used herein, refers to either RNA or
25 DNA that encodes a specific amino acid sequence or its complementary strand. A polynucleotide may also not encode a polypeptide, such as, for example, an antisense oligonucleotide, or a ribozyme. The DNA or RNA may be single stranded or double stranded.

The term a "chimeric polynucleotide" as used herein refers to a polynucleotide sequence that includes part of the coding sequences of more than one protein where the proteins are not naturally encoded in close proximity to one another on the same nucleic acid molecule. To form the chimeric polynucleotide, the coding sequences of two or more proteins are isolated and linked by techniques commonly practiced in the art of recombinant DNA technology. The chimeric polynucleotide molecule will contain one or more regulatory sequences for expression of the chimera in appropriate host cells, or in vivo. The regulatory sequences can be naturally associated with one of the coding sequences or may be heterologous to one or more of the coding sequences. Each coding sequence may have its own regulatory sequences. The chimeric polynucleotide expression product is a chimera of portions of two not-naturally associated polypeptides.

The term "chimera of a ligand and a viral surface protein" as used herein refers to the expression product of a chimeric polynucleotide that encodes a portion of a ligand and a portion of a viral surface protein. The chimera is constructed so that the ligand portion can bind the receptor that the ligand binds in the natural environment, so that the chimeric viral surface protein can incorporate into a viral surface to form a chimeric viral envelope, and so that the chimeric viral surface can surround a viral genome much as in the natural environment. Thus, the chimera is a dual functioning polypeptide or a multi-functioning polypeptide, retaining at least the function of the ligand and the function of the viral surface protein that comprise it.

The term "incorporation aggregate" as used herein refers to the phenomenon where a virus or particle is surrounded or encapsulated by a surface protein to form a vesicle surrounded or incorporated by the surface protein. The term incorporation aggregate describes a pool of surface proteins or chimeric surface polypeptides that function to incorporate a virus or particle into a vesicle. The vesicle is then defined in part by the qualities of the surface protein into which it is incorporated. The vesicle can be a virus or a particle, and can be internalized in a target cell based on the specificity of the surface protein by which it is incorporated.

The term "regulatory sequence" refers to a nucleic acid sequence encoding one or more elements that are capable of affecting or effecting expression of a gene sequence, including transcription or translation thereof, when the gene sequence is placed in such a position as to subject it to the control thereof. Such a regulatory sequence can be, for example, a minimal promoter sequence, a complete promoter sequence, an enhancer sequence, an upstream activation sequence ("UAS"), an operator sequence, a downstream termination sequence, a polyadenylation sequence, an optimal 5' leader sequence to optimize initiation of translation, and a Shine-Dalgarno sequence. Alternatively, the regulatory sequence can contain a combination enhancer/promoter element. The regulatory sequence that is appropriate for expression of the gene of interest differs depending upon the host system in which the construct is to be expressed. Selection of the appropriate regulatory sequences for use herein is within the capability of one skilled in the art. In eukaryotes, for example, such a sequence can include one or more of a promoter sequence and/or a transcription termination sequence. Regulatory sequences suitable for use herein may be derived from any source including a prokaryotic source, an eukaryotic source, a virus, a viral vector, a bacteriophage or a linear or circular plasmid. The regulatory sequence herein can also be a synthetic sequence, for example, one made by combining the UAS of one gene with the remainder of a requisite promoter from another gene, such as the GAPDH/ADH2 hybrid promoter, as described in U.S. Patents number 4,876,197 and 4,880,734. The regulatory sequences suitable for use herein can be any regulatory sequence that is compatible for use with the promoters for expression in a desired host cell. For example, for expression in yeast, a regulatory sequence derived from yeast systems would be desirable. The regulatory sequence can be a sequence naturally associated with the promoters selected for use herein, or can be a synthetic sequence, or partly synthetic or partly derived. The promoters suitable for use herein can be any promoter, including those that are constitutively active or those that are inducible or regulatable. The promoters can be naturally derived or synthetically made. They can be derived from any genes, viral, prokaryotic or eukaryotic. The eukaryotic genes can be

yeast or other fungal, insect, mammalian or avian genes. Examples of suitable promoters are described below in the portion relating to expression systems.

The terms "protein" or "polypeptide" or "peptide" used herein in the context of an element of the invention include the product of a gene expressed or regulated. The protein, polypeptide, or peptide of the invention may be the ligand or the portion of the ligand that makes up a chimeric polypeptide for surrounding a virus and binding to a cell surface receptor on cells targeted for viral infection and viral replication of heterologous genes. The protein, polypeptide, or peptide may be a portion of the viral surface that makes up the chimeric polypeptide for surrounding a virus and binding to a cell surface receptor on cells targeted for viral infection. The protein, polypeptide or peptide of the invention may also be the non-viral polypeptide expressed by the non-viral coding sequence present in the viral genome for expression in virally infected cells. The terms "protein", "polypeptide" or "peptide" as used herein also includes "mature protein" and "analogs" thereof and "portions" thereof that are truncations, variants, alleles and derivatives of the mature protein.

The term "a portion of" as used herein in the context of "a portion of a ligand" and "a portion of a viral surface protein" refers to a portion of the entire coding sequence or expression product of a biologically active ligand or viral surface protein, the portion also retaining the biological activity of the ligand or the viral surface protein. Biological activity is, for example, in the case of the ligand, ligand binding; and in the case of the viral surface protein is, for example, the ability to surround, envelop, or encapsulate a virus or particle. Biological activity of a viral surface protein is also indicated by the ability of the viral surface protein to become anchored in the membrane that encapsulates the virus or particle. As with the term protein or polypeptide, the term "a portion of" as used herein also refers to portions of mature proteins and analogs thereof. The analogs may be truncations, variants, alleles, and derivatives of the mature protein. Unless specifically mentioned otherwise, the "analogs" possess one or more of the bioactivities of the "mature protein." Thus, polypeptides that are identical or contain at least 60%, preferably 70%, more preferably 80%, and most preferably 90% amino acid sequence

homology to the amino acid sequence of the mature protein wherever derived, from human or nonhuman sources, are included within this definition.

The term "derived from" as used herein indicates, in the case of the portion of a viral surface protein that is "derived from a viral surface of" a virus, that the viral surface
5 protein portion used to construct the chimeric viral surface protein of the invention has high sequence homology to the viral surface protein of a native virus.

The term "variants" as used herein refers to "variants of a protein" or "variants of a polypeptide" or "variants of a portion of a protein or a polypeptide". The term "variants" as used herein refers to proteins or polypeptides or portions of proteins or polypeptides
10 that contain amino acid substitutions, deletions, or insertions. The amino acid substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acid residues such as to alter a glycosylation site, a phosphorylation site, an acetylation site, or to minimize misfolding by substitution or deletion of one or more cysteine residues that are not necessary for function. Conservative amino acid
15 substitutions are those that preserve the general charge, hydrophobicity/hydrophilicity and/or steric bulk of the amino acid substituted, for example, substitutions between the members of the following groups are conservative substitutions: Gly/Ala, Val/Ile/Leu, Asp/Glu, Lys/Arg, Asn/Gln, and Phe/Trp/Tyr. The analogs herein further include peptides having one or more peptide mimics, also known as peptoids, that possess the bioactivity of
20 the protein. Also included within the definition are polypeptides containing one or more analog amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. The term polypeptide also does not exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations,
25 phosphorylations and the like.

The term "ligand" as used herein refers to a molecule or complex of molecules that bind a receptor, such as a cell surface protein receptor. A ligand can be a peptide, polypeptide, protein, any other molecule capable of forming a binding pair with a receptor, or any complex of more than one molecule capable of the same. The ligand

can also be a portion of a ligand such that the binding portion of the ligand is retained and the portion of the ligand can still bind the receptor. The ligand can also be a portion of a complex that binds the receptor, the portion retaining the ability to bind the receptor and the ability to remain bound to the polypeptide or polypeptides of the
5 complex. If the ligand is a ligand complex it can comprise a polypeptide complexed to at least one other polypeptide and forming a binding site for a receptor. The binding between the ligand or the ligand complex and receptor is characterized as high affinity in order that a binding pair is formed; the binding pair may form a non-covalent or covalent bond. Examples of a ligand complex binding a receptor exist with the SERPIN
10 PAI-1 bound to uPA or tPA. In the native environment uPA is bound to uPAR on the cell surface. uPA will bind PAI-1 covalently and forms a complex separate from uPAR. The complex uPA:PAI-1 binds LRP, also on the cell surface. LRP may bind at PAI-1 of the complex or at both PAI-1 and uPA. The binding preceeds internalization of the complex into the cell by an LRP mediated mechanism. The ligand is part of a ligand
15 complex, the ligand portion retains at least the ability to bind and the polypeptide or polypeptides of the ligand complex, and also at least the ability to bind the receptor of the LDL-receptor family for which it is specific.

The term "receptor" as used herein refers to a protein molecule that binds a ligand to form a binding pair. Receptors are expressed on the cell surface. Binding of a ligand
20 to a receptor often initiates a cellular response, such as for example, internalization of the ligand:receptor binding pair, or aggregation of receptors on the cell surface. Examples of cell-surface receptors include receptors of the LDL-receptor family, receptors that bind SERPIN:protease complexes, receptors that bind and internalize PAI-1 and complexes bound to PAI-1, the LRP receptor, the gp330 receptor, and receptors
25 that bind and internalize TFPI.

The term "LDL-receptor family" as used herein refers to the family of low density lipoprotein receptors. The family includes, but is not limited to, the low density lipoprotein receptor (LDLR), the very low density lipoprotein receptor-1 and very low density lipoprotein receptor -2 (VLDLR-1 and VLDLR-2), the low density lipoprotein-

receptor related protein (LRP), glycoprotein 330, and a gp-330-like protein identified in *Caenorhabditis elegans* (*C. elegans*) as disclosed in Yochem and Greenwald, *Proc. Natl. Acad. Sci. USA* 90:4572-4576, (1993). These receptors and their function are disclosed generally in a review article, and the references cited therein, by Strickland *et al*, *FASEB J.* 5 9:890-898, (1995).

The term "SERPIN" as used herein refers to a class of proteins that are serine protease inhibitors. The classification includes such proteins as, for example, PAI-1, α -1-antitrypsin and α -1-antichymotrypsin.

"PAI-1" as used herein refers to plasminogen activator inhibitor - 1. Isolation and 10 cloning of PAI-1 is described in Pannekoek *et al.*, *EMBO*, 5:2539-2544 (1986).

"tPA" as used herein refers to tissue type plasminogen activator, a protease capable of activating tissue type plasminogen.

"uPA" as used herein refers to urokinase plasminogen activator, a protease capable of activating urokinase plasminogen.

15 "uPAR" as used herein refers to the urokinase plasminogen activator receptor. uPAR is a cell surface receptor that binds urokinase plasminogen activator (uPA).

"TFPI" as used herein refers to tissue factor pathway inhibitor.

"PSA" as used herein refers to prostate specific antigen.

The term "a binding pair" as used herein refers to a pair of molecules, usually 20 referring to a protein/protein pair, but does not exclude a protein/DNA pair, or a protein/RNA pair in which the components of the pair bind specifically to each other with a higher affinity than to a random molecule, such that upon binding, for example, in case of a ligand/receptor interaction, the binding pair triggers a cellular or an intracellular response. The ligand can be one polypeptide molecule, or can be a polypeptide molecule 25 complexed to one or more polypeptide molecules to form a ligand complex. The ligand complex can bind a receptor and form a binding pair. A key aspect of the invention is that the ligand for the receptors of the invention are specific for ligand complexes of more than one polypeptide, in addition to being specific for single polypeptide ligand. It is believed that the specificity of internalization which is an object of the invention is created in part

by ligands that comprises more than one polypeptide complexed together capable of binding a receptor of the LDL-receptor family. The binding pair is formally the ligand or the ligand complex, and the binding pair is formed by the complex and the receptor when binding occurs. The binding sites of the binding pair may be on any part of the ligand, or the ligand complex. Thus, for example, the receptor may bind part of both polypeptides of a two polypeptide ligand complex, or may only bind one of the polypeptides of the complex. The binding pair may be formed by the formation of a covalent bond between the ligand and the receptor, or the ligand complex and the receptor. The response that results from the forming of the binding pair can include, for example, internalization of the ligand or the ligand complex bound to the cell surface receptor. The binding pair between a receptor and a ligand or ligand complex can also be aided by the association of another receptor or another polypeptide molecule for facilitation of the subsequent cellular response, such as for example, internalization of the ligand complex/receptor binding pair. An example of a ligand/receptor binding pair is a pair formed between PDGF (platelet derived growth factor) and the PDGF receptor, a cell surface receptor. Another example of a binding pair is the ligand complex u-PA:PAI-1 that binds LRP on a cell surface. An example of a different binding pair is an antigen/antibody pair in which the antibody is generated by immunization of a host with the antigen. Specific binding indicates a binding interaction having a low dissociation constant, which distinguishes specific binding from non-specific, background, binding.

The term "viral surface protein" as used herein refers to the protein or polypeptide that makes up the viral surface of a virus. Viral surface proteins useful for the invention include those surface proteins from enveloped viruses, including retroviruses, and non-enveloped viruses, including, for example, adenovirus. Thus, the viral surface proteins may be selected from any viral surface protein, including capsid proteins, from, for example an adeno-virus, an adeno-associated virus, a herpes simplex virus, a cytomegalovirus, a retrovirus, an influenza virus, a sindbis virus, and a vesicular stomatitis virus, a retrovirus, herpes simplex virus, cytomegalovirus, and human immune deficiency virus.

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The term "internalization of the virus" or "internalization of the particle" as used herein refers to internalization of a virus or a particle or a vesicle into host cell by contact of a receptor on the cell surface with the ligand portion of the viral surface protein or by contact of the receptor on the cell surface with the ligand that is affixed to the particle.

- 5 The virus or particle is then internalized after a binding pair is formed between the receptor and the ligand portion of either the viral surface protein or the altered particle, as the case may be.

The term a "particle" as used herein refers to any vesicle that can encapsulate a polynucleotide coding sequence, including also regulatory sequences, for expression of the
10 coding sequence in a host cell. Particles can be, for example, viral particles or non-viral particles. Viral particles can be any enveloped or non-enveloped viral particle and can include a viral genome and heterologous polynucleotide sequences. Exemplary viral particles can be derived from adeno-virus, an adeno-associated virus, herpes simplex virus, cytomegalovirus, a retrovirus, an influenza virus, a sindbis virus, semliki forest virus and a
15 vesicular stomatitis virus. Exemplary nonviral particles include liposomes such as, for example, those disclosed in U.S. Patent No. 5,422,120, WO 95/13796, WO 94/23697, WO 91/14445 and EP 524.968 B1, particularly including heterovesicular liposomal particles.
MAYBE ADD SOME REFERENCE TO HBV, 36 SERIES - BARB'S POINT 7 -
DOUBLE CHECK.

- 20 The term an "altered particle" as used herein refers to any vesicle or particle that has been altered with the addition of a ligand portion such that the ligand portion can form a binding pair with a receptor of the LDL-receptor family. The ligand portion may also be connected to a viral surface protein portion. The ligand can be any ligand that binds a member of the LDL-receptor family. The ligand may be the entire ligand or a biologically
25 active portion thereof, such that the ligand portion retains the ability to form a binding pair with the receptor. The portion of the ligand is affixed to the particle or vesicle by means of affixing peptides or polypeptides to non-protein molecules standard in the art, including but not limited to chemical or UV light crosslinking. The polynucleotide coding region may be incorporated into the particle or vesicle before or after the ligand portion is affixed

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to the particle or vesicle. The altered particle containing a coding sequence and regulatory sequences for expression in a host cell, is internalized into a host cell when the ligand affixed to the particle forms a binding pair with the receptor present on the host cell surface.

5 Exemplary expression systems are described below for the construction of recombinant coding sequences in vectors for incorporation into a viral genome or for incorporation into a particle or vesicle and for internalization into a host cell in an animal for expression in the animal. In addition, the expression systems are useful for the construction of the chimeric polynucleotide encoding a chimeric viral surface protein
10 comprising portions of a ligand and a viral surface protein, and the expression of the chimeric viral surface protein. The expression systems below are also useful for the construction of the coding region of a portion of a ligand that is expressed and affixed to a particle or vesicle for internalization of the particle or vesicle by host cell expressing the receptor for the ligand. The ligand portion may be a peptide or a polypeptide, and retains
15 the biological activity of forming a binding pair with the receptor that the ligand from which it was derived binds in the natural environment.

Although the methodology described below is believed to contain sufficient details to enable one skilled in the art to practice the present invention, other items not specifically exemplified, such as plasmids, can be constructed and purified using
20 standard recombinant DNA techniques described in, for example, Sambrook *et al.* (1989), MOLECULAR CLONING: A LABORATORY MANUAL, 2d edition (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.), and Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (1994), (Greene Publishing Associates and John Wiley & Sons, New York, N.Y.). under the current regulations described in United
25 States Dept. of HHS, NATIONAL INSTITUTE OF HEALTH (NIH) GUIDELINES FOR RECOMBINANT DNA RESEARCH. These references include procedures for the following standard methods: cloning procedures with plasmids, transformation of host cells, cell culture, plasmid DNA purification, phenol extraction of DNA, ethanol

precipitation of DNA, agarose gel electrophoresis, purification of DNA fragments from agarose gels, and restriction endonuclease and other DNA-modifying enzyme reactions.

Expression in Bacterial Cells

Control elements for use in bacteria include promoters, optionally containing operator sequences, and ribosome binding sites. Useful promoters include sequences derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*), the β -lactamase (*bla*) promoter system, bacteriophage λ PL, and T7. In addition, synthetic promoters can be used, such as the *tac* promoter. The β -lactamase and lactose promoter systems are described in Chang *et al.*, *Nature* (1978) 275: 615, and Goeddel *et al.*, *Nature* (1979) 281: 544; the alkaline phosphatase, tryptophan (*trp*) promoter system are described in Goeddel *et al.*, *Nucleic Acids Res.* (1980) 8: 4057 and EP 36,776 and hybrid promoters such as the *tac* promoter is described in U.S. Patent No. 4,551,433 and deBoer *et al.*, *Proc. Natl. Acad. Sci. USA* (1983) : 21-25. However, other known bacterial promoters useful for expression of eukaryotic proteins are also suitable. A person skilled in the art would be able to operably ligate such promoters to the coding sequences of interest, for example, as described in Siebenlist (1980) : 269, using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (SD) sequence operably linked to the DNA encoding the target polypeptide. For prokaryotic host cells that do not recognize and process the native target polypeptide signal sequence, the signal sequence can be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, Ipp, or heat stable enterotoxin II leaders. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria.

The foregoing systems are particularly compatible with .
However, numerous other systems for use in bacterial hosts including Gram-negative or Gram-positive organisms such as , *S* , *S* ,
species such as *S* or *S*

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among others Methods for introducing exogenous DNA into these hosts typically include the use of CaCl_2 or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation, nuclear injection, or protoplast fusion as described generally in Sambrook (1989), cited above These examples are illustrative rather than limiting. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

Prokaryotic cells used to produce the target polypeptide of this invention are cultured in suitable media, as described generally in Sambrook cited above.

10

Expression in Yeast Cells

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for, among others, the following

15 yeasts: *S*, as described in Hinnen *A S*
USA (1978) : 1929; Ito (1983) : 163; as
described in Kurtz (1986) : 142; as described
in Kunze (1985) : 141; as
described in Gleeson (1986) : 3459 and Roggenkamp
20 (1986) : 302); as described in Das
(1984) : 1165; as described in De
Louvencourt (1983) : 737 and Van den Berg
(1990) : 135; as described in Kunze
(1985) : 141; as described in Cregg
25 (1985) : 3376 and U.S. Patent Nos. 4,837,148 and 4,929,555;
S as described in Beach and Nurse, (1981) : 706;
and as described in Davidow (1985) : 380
and Gaillardin (1985) : 49, *A* hosts such as *A*.
as described in Ballance *Biochem. Biophys. Res. Commun.* (1983) : 284-289;

Tilburn *et al.* *Gene* (1983) : 205-221 and Yelton *et al.* *Proc. Natl. Acad. Sci. USA* (1984) : 1470-1474, and *A. nidulans* as described in Kelly and Hynes, *EMBO J.* (1985) : 475479; *richoderma reesia* as described in EP 244,234, and filamentous fungi such as, e.g., *Neurospora* *Penicillium* *obypocladium* as described in WO 91/00357.

5 Control sequences for yeast vectors are known and include promoters regions from genes such as alcohol dehydrogenase (ADH), as described in EP 284,044, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK), as described in EP 329,203. The yeast *P O* gene, encoding
10 acid phosphatase, also provides useful promoter sequences, as described in Myanohara *et al.*, *Proc. Natl. Acad. Sci. USA* (1983) : 1. Other suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase, as described in Hitzeman *et al.*, *J. Biol. Chem.* (1980) : 2073, or other glycolytic enzymes, such as pyruvate decarboxylase, triosephosphate isomerase, and phosphoglucose isomerase,
15 as described in Hess *et al.*, *J. Ad. En yme Re.* (1968) : 149 and Holland *et al.*, *Biochemistry* (1978) : 4900. Inducible yeast promoters having the additional advantage of transcription controlled by growth conditions, include from the list above and others the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein,
20 glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EP 073,657. Yeast enhancers also are advantageously used with yeast promoters. In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, upstream activating sequences
25 (UAS) of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region, as described in U.S. Patent Nos. 4,876,197 and 4,880,734. Other examples of hybrid promoters include promoters which consist of the regulatory

sequences of either the *A*, *GA*, *GA*, or *PO* genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as *GAP* or *Py*, as described in EP 164,556. Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription.

Other control elements which may be included in the yeast expression vectors are terminators, for example, from *GAP* and from the enolase gene, as described in Holland *et al.*, *J. Biol. Chem.* (1981) : 1385, and leader sequences which encode signal sequences for secretion. DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast invertase gene as described in EP 012,873 and JP 62,096,086 and the α -factor gene, as described in U.S. Patent Nos. 4,588,684, 4,546,083 and 4,870,008; EP 324,274; and WO 89/02463. Alternatively, leaders of non-yeast origin, such as an interferon leader, also provide for secretion in yeast, as described in EP 060,057.

Methods of introducing exogenous DNA into yeast hosts are well known in the art, and typically include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations.

Transformations into yeast can be carried out according to the method described in Van Solingen *et al.*, *J. Bact.* (1977) :946 and Hsiao *et al.*, *Proc. Natl. Acad. Sci. USA* (1979) :3829. However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used as described generally in Sambrook *et al.*, cited above.

For yeast secretion the native target polypeptide signal sequence may be substituted by the yeast invertase, α -factor, or acid phosphatase leaders. The origin of replication from the 2 μ plasmid origin is suitable for yeast. A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid described in Kingsman *et al.*, *Gene* (1979) : 141 or Tschemper *et al.*, *Gene* (1980) 157. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in

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tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* Gene.

For intracellular production of the present polypeptides in yeast, a sequence encoding a yeast protein can be linked to a coding sequence of the polypeptide to
5 produce a fusion protein that can be cleaved intracellularly by the yeast cells upon expression. An example, of such a yeast leader sequence is the yeast ubiquitin gene.

Expression in Insect Cells

Baculovirus expression vectors (BEVs) are recombinant insect viruses in which
10 the coding sequence for a foreign gene to be expressed is inserted behind a baculovirus promoter in place of a viral gene, e.g., polyhedrin, as described in Smith and Summers, U.S. Pat. No., 4,745,051.

An expression construct herein includes a DNA vector useful as an intermediate for the infection or transformation of an insect cell system, the vector generally
15 containing DNA coding for a baculovirus transcriptional promoter, optionally but preferably, followed downstream by an insect signal DNA sequence capable of directing secretion of a desired protein, and a site for insertion of the foreign gene encoding the foreign protein, the signal DNA sequence and the foreign gene being placed under the transcriptional control of a baculovirus promoter, the foreign gene herein being the
20 coding sequence of the polypeptide.

The promoter for use herein can be a baculovirus transcriptional promoter region derived from any of the over 500 baculoviruses generally infecting insects, such as, for example, the Orders Lepidoptera, Diptera, Orthoptera, Coleoptera and Hymenoptera including, for example, but not limited to the viral DNAs of *Autographa californica*
25 MNPV, *Bomby mori* NPV, *Trichoplusia ni* MNPV, *Rachlplusia ou* MNPV or *Galleria mellonella* MNPV, *Aedes aegypti*, *Syngrapha melanosoma*, *Spodoptera frugiperda*, and *Trichoplusia ni*. Thus, the baculovirus transcriptional promoter can be, for example, a baculovirus immediate-early gene IEL or IEN promoter; an immediate-early gene in combination with a baculovirus delayed-early gene promoter region selected

from the group consisting of a 39K and a *indIII* fragment containing a delayed-early gene; or a baculovirus late gene promoter. The immediate-early or delayed-early promoters can be enhanced with transcriptional enhancer elements.

Particularly suitable for use herein is the strong polyhedrin promoter of the
 5 baculovirus, which directs a high level of expression of a DNA insert, as described in Friesen *et al.* (1986) "The Regulation of Baculovirus Gene Expression" in: THE MOLECULAR BIOLOGY OF BACULOVIRUSES (W.Doerfler, ed.); EP 127,839 and EP 155,476; and the promoter from the gene encoding the p10 protein, as described in Vlak *et al.*, *J. Gen. irol.* (1988) :765-776.

10 The plasmid for use herein usually also contains the polyhedrin polyadenylation signal, as described in Miller *et al.*, *Ann. Re. Micro iol.* (1988) :177 and a procaryotic ampicillin-resistance (*amp*) gene and an origin of replication for selection and propagation in *E. coli*. DNA encoding suitable signal sequences can also be included and is generally derived from genes for secreted insect or baculovirus proteins,
 15 such as the baculovirus polyhedrin gene, as described in Carbonell *et al.*, *Gene* (1988) :409, as well as mammalian signal sequences such as those derived from genes encoding human α -interferon as described in Maeda *et al.*, *Nature* (1985) :592-594; human gastrin-releasing peptide, as described in Lebacq-Verheyden *et al.*, *Mol. Cell. Biol.* (1988) : 3129; human IL-2, as described in Smith *et al.*, *Proc. Natl. Acad. Sci. USA* (1985) :8404; mouse IL-3, as described in Miyajima *et al.*, *Gene* (1987) :273;
 20 and human glucocerebrosidase, as described in Martin *et al.*, *NA* (1988) 7:99.

Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera ru iperda* (caterpillar), *Aedes ae ypti* (mosquito), *Aedes al opictus* (mosquito), *rosophila melano aster* (fruitfly), and
 25 *Bom y mori* host cells have been identified and can be used herein. See, for example, the description in Luckow *et al.*, *Bio/Technology*(1988) : 47-55, Miller *et al.*, in GENETIC ENGINEERING (Setlow, J.K. *et al.* eds.), Vol. 8 (Plenum Publishing, 1986), pp. 277-279, and Maeda *et al.*, *Nature*, (1985) : 592-594. A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa cali ornica* NPV

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and the Bm-5 strain of *Bomby mori* NPV. Such viruses may be used as the virus for transfection of host cells such as *Spodoptera rugiperda* cells.

Other baculovirus genes in addition to the polyhedrin promoter may be employed to advantage in a baculovirus expression system. These include immediate-early (alpha), delayed-early (beta), late (gamma), or very late (delta), according to the phase of the viral infection during which they are expressed. The expression of these genes occurs sequentially, probably as the result of a "cascade" mechanism of transcriptional regulation. Thus, the immediate-early genes are expressed immediately after infection, in the absence of other viral functions, and one or more of the resulting gene products induces transcription of the delayed-early genes. Some delayed-early gene products, in turn, induce transcription of late genes, and finally, the very late genes are expressed under the control of previously expressed gene products from one or more of the earlier classes. One relatively well defined component of this regulatory cascade is IEI, a preferred immediate-early gene of *Autographa californica* nuclear polyhedrosis virus (AcMNPV). IEI is expressed in the absence of other viral functions and encodes a product that stimulates the transcription of several genes of the delayed-early class, including the preferred 39K gene, as described in Guarino and Summers, *J. virol.* (1986) 7:563-571 and *J. virol.* (1987) 61:2091-2099 as well as late genes, as described in Guanno and Summers, *J. virol.* (1988) 62:444-451.

Immediate-early genes as described above can be used in combination with a baculovirus gene promoter region of the delayed-early category. Unlike the immediate-early genes, such delayed-early genes require the presence of other viral genes or gene products such as those of the immediate-early genes. The combination of immediate-early genes can be made with any of several delayed-early gene promoter regions such as 39K or one of the delayed-early gene promoters found on the *indIII* fragment of the baculovirus genome. In the present instance, the 39 K promoter region can be linked to the foreign gene to be expressed such that expression can be further controlled by the presence of IEI, as described in L. A. Guarino and Summers (1986a),

cited above; Guarino & Summers (1986b) *J. virol.*, (1986) :215-223, and Guarino *et al.* (1986c), *J. virol.* (1986) :224-229.

Additionally, when a combination of immediate-early genes with a delayed-early gene promoter region is used, enhancement of the expression of heterologous genes can be realized by the presence of an enhancer sequence in direct cis linkage with the delayed-early gene promoter region. Such enhancer sequences are characterized by their enhancement of delayed-early gene expression in situations where the immediate-early gene or its product is limited. For example, the hr5 enhancer sequence can be linked directly, in cis, to the delayed-early gene promoter region, 39K, thereby enhancing the expression of the cloned heterologous DNA as described in Guarino and Summers (1986a), (1986b), and Guarino *et al.* (1986).

The polyhedrin gene is classified as a very late gene. Therefore, transcription from the polyhedrin promoter requires the previous expression of an unknown, but probably large number of other viral and cellular gene products. Because of this delayed expression of the polyhedrin promoter, state-of-the-art BEVs, such as the exemplary BEV system described by Smith and Summers in, for example, U.S. Pat. No., 4,745,051 will express foreign genes only as a result of gene expression from the rest of the viral genome, and only after the viral infection is well underway. This represents a limitation to the use of existing BEVs. The ability of the host cell to process newly synthesized proteins decreases as the baculovirus infection progresses. Thus, gene expression from the polyhedrin promoter occurs at a time when the host cell's ability to process newly synthesized proteins is potentially diminished for certain proteins such as human tissue plasminogen activator. As a consequence, the expression of secretory glycoproteins in BEV systems is complicated due to incomplete secretion of the cloned gene product, thereby trapping the cloned gene product within the cell in an incompletely processed form.

While it has been recognized that an insect signal sequence can be used to express a foreign protein that can be cleaved to produce a mature protein, the present

invention is preferably practiced with a mammalian signal sequence appropriate for the gene expressed.

An exemplary insect signal sequence suitable herein is the sequence encoding for a Lepidopteran adipokinetic hormone (AKH) peptide. The AKH family consists of short
5 blocked neuropeptides that regulate energy substrate mobilization and metabolism in insects. In a preferred embodiment, a DNA sequence coding for a Lepidopteran *Manduca sexta* AKH signal peptide can be used. Other insect AKH signal peptides, such as those from the Orthoptera *Schistocerca gregaria* locus can also be employed to advantage. Another exemplary insect signal sequence is the sequence coding for
10 *Drosophila* cuticle proteins such as CPI, CP2, CP3 or CP4.

Currently, the most commonly used transfer vector that can be used herein for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, can also be used herein. Materials and methods for baculovirus/insect cell expression systems are commercially available in a kit form from companies such as
15 Invitrogen (San Diego CA) ("MaxBac" kit). The techniques utilized herein are generally known to those skilled in the art and are fully described in Summers and Smith, A MANUAL OF METHODS FOR BACULOVIRUS VECTORS AND INSECT CELL CULTURE PROCEDURES, Texas Agricultural Experiment Station Bulletin No. 1555, Texas A&M University (1987); Smith *et al.*, *Mol. Cell. Biol.* (1983) 3: 2156, and
20 Luckow and Summers (1989). These include, for example, the use of pVL985 which alters the polyhedrin start codon from ATG to ATT, and which introduces a *Bam*HI cloning site 32 basepairs downstream from the ATT, as described in Luckow and Summers, *irology* (1989) 7:31.

Thus, for example, for insect cell expression of the present polypeptides, the
25 desired DNA sequence can be inserted into the transfer vector, using known techniques. An insect cell host can be cotransformed with the transfer vector containing the inserted desired DNA together with the genomic DNA of wild type baculovirus, usually by cotransfection. The vector and viral genome are allowed to recombine resulting in a

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recombinant virus that can be easily identified and purified. The packaged recombinant virus can be used to infect insect host cells to express a desired polypeptide.

Other methods that are applicable herein are the standard methods of insect cell culture, cotransfection and preparation of plasmids are set forth in Summers and Smith
5 (1987), cited above. This reference also pertains to the standard methods of cloning genes into AcMNPV transfer vectors, plasmid DNA isolation, transferring genes into the AcMNPV genome, viral DNA purification, radiolabeling recombinant proteins and preparation of insect cell culture media. The procedure for the cultivation of viruses and cells are described in Volkman and Summers, *J. virol.* (1975) :820-832 and
10 Volkman, *al.*, *J. virol.* (1976) :820-832.

Expression in Mammalian Cells

Typical promoters for mammalian cell expression of the polypeptides of the invention include the SV40 early promoter, the CMV promoter, the mouse mammary
15 tumor virus LTR promoter, the adenovirus major late promoter (Ad MLP), and the herpes simplex virus promoter, among others. Other non-viral promoters, such as a promoter derived from the murine metallothionein gene, will also find use in mammalian constructs. Mammalian expression may be either constitutive or regulated (inducible), depending on the promoter. Typically, transcription termination and
20 polyadenylation sequences will also be present, located 3' to the translation stop codon. Preferably, a sequence for optimization of initiation of translation, located 5' to the polypeptide coding sequence, is also present. Examples of transcription terminator/polyadenylation signals include those derived from SV40, as described in Sambrook et al. (1989), cited previously. Introns, containing splice donor and acceptor
25 sites, may also be designed into the constructs of the present invention.

Enhancer elements can also be used herein to increase expression levels of the mammalian constructs. Examples include the SV40 early gene enhancer, as described in Dijkema *et al.*, *EMBO J.* (1985) :761 and the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, as described in Gorman *et al.*,

Proc. Natl. Acad. Sci. USA (1982b) 79:6777 and human cytomegalovirus, as described in Boshart *et al.*, *Cell* (1985) :521. A leader sequence can also be present which includes a sequence encoding a signal peptide, to provide for the secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the gene of interest such that the leader sequence can be cleaved either *in vivo* or *in vitro*. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

Once complete, the mammalian expression vectors can be used to transform any of several mammalian cells. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Patent No. 4,399,216.

Practice of the Invention

General Description

It was recognized herein that cells can be targeted with viruses or particles containing molecular contents for delivery in a cell or tissue specific manner. The molecular contents include genes for expression in target cells and also include therapeutics or other molecules. Herein are described chimeric viral surface polypeptides including a portion of a ligand for binding to a cell surface receptor of the LDL-receptor family and a portion of a viral surface protein for incorporation of a virus or particle.

According to the invention, a virus or particle is incorporated by the chimeric viral surface polypeptides and used to target cells expressing receptors that bind the ligand portion of the chimera. The virus that is incorporated may be any virus, including but not limited to an adeno-virus, an adeno-associated virus, a retrovirus, a sindbis virus, and a BK virus. The viral genomes of these viruses are particularly well

suited to expression of heterologous polypeptides in target cells, but any viral genome capable of expressing heterologous polypeptides in target cells is contemplated by the invention. The particles that are incorporated and internalized can be any particle capable of containing and delivering molecular contents to a target cell. Examples of particles useful for the invention include, but are not limited to, for example, liposomes, including heterovesicular liposomes and cyclodextrin liposomes. The liposomes are also useful in the invention for construction of altered particles described below. Once the ligand portion of the chimera binds to the cell surface receptor, the virus or particle is internalized, presumably via a receptor/ligand internalization mechanism. Once inside the cell, the contents of the virus or particle are released. However, the invention is not limited to any theories of how the invention works.

Example 1

Design of Chimeric Viral Surface Polypeptides

The chimeric viral surface polypeptide of the invention is composed of two portions: a viral surface protein portion and a ligand portion. The viral surface protein portion of the invention can be derived from any virus, enveloped or non-enveloped. The viral surface protein portion of the chimera can be derived from, for example, surface proteins of an adeno-virus, an adeno-associated virus, a herpes simplex virus, a cytomegalovirus, a retrovirus, an influenza virus, a sindbis virus, a semliki forest virus or a vesicular stomatitis virus. The viral surface protein portion can be the entire viral surface protein, or a portion of it. The portion of the viral surface protein includes that portion that allows the surface protein to incorporate into a virus or particle.

The ligand portion of the chimera is derived from a ligand specific for a receptor of the LDL-receptor family. The ligand portion can be composed of all of the ligand or only a portion of it. The essential part of the ligand is the portion of the ligand that binds the receptor or that allows the receptor-ligand interaction to occur. The chimera is constructed so that binding of the ligand to the receptor is possible and is retained to some extent from the native molecule. The ligand is selected, for example, from the following known ligands of LDL-receptors, including, but not limited to serine protease inhibitors

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(SERPINs), including, for example, PAI-1, protease nexin 1, α -1-antitrypsin and α -1-antichymotrypsin. The ligand can also be, for example, TFPI, and other ligands known to bind a receptor of the LDL-receptor family.

The ligand of the invention may also be defined in terms of a ligand complex of more than one polypeptide complexed together and capable of binding as a complex to a receptor of the LDL-receptor family. Examples of such complexes include PAI-1 bound to uPA, PAI-1 bound to tPA, and α -1-antitrypsin bound to PSA. The PAI-1:uPA complex binds, for example, LRP on a cell surface. In the case where a ligand complex forms the basis of the ligand portion of the invention, the ligand portion comprises the parts of the ligand complex that are necessary for binding to the receptor and for internalization of the complex by the receptor-mediated mechanism of internalization into the cell.

Example 2

The Chimeras are Specific for a Cell Surface Protein or Complex

In the typical cases, prior to this invention, a ligand is expressed on the virus or other gene delivery particle and this binds to and is internalized by a cell expressing the receptor. The specificity of this event is dependent on the amount and distribution of the receptor on the various cell types, including the target cell. In this case, if the receptor already has the ligand bound to it, eg., erythropoietin bound to the EPO receptor, then a gene delivery particle is not able to bind to that cell. In the case of the invention described herein, the gene delivery particle is decorated with a ligand, which recognizes a ligand:receptor complex. In a particular embodiment of the invention, a gene delivery particle expressing PAI-1, will recognize and to bind covalently to active two chain uPA bound to the uPAR receptor. In this way only cells which are actively involved in cell surface plasminogen activation, and are displaying active receptor bound uPA, will be targeted by the gene delivery particle. After formation of the particle:uPA:uPAR complex, efficient internalization of the particle will occur via transfer to LRP or related scavenger receptors. The invention thus achieves enhanced specificity for target cells.

Example 3

Construction of the Chimeric Viral Surface Polypeptide

Construction of the chimeric viral surface polypeptide is achieved by employing techniques known in the art of recombinant DNA technology. Where the chimera is expressed in a cell infected with a virus, the expression of the chimera is followed by viral budding from the cell, and results in a virus incorporated by the chimera and displaying the chimera on the envelope of an external portion of the virus. Where the chimera is constructed independently and used to form a composition that can incorporate into a virus or particle, the chimera can be constructed and expressed by any expression system known in the art, including, for example, those disclosed herein. Once the chimera is expressed, it is used to form a composition that is mixed with a viral particle, or other particle for incorporation of the virus or particle by the chimera.

Example 4

The Incorporated Particle is Exposed to Target Cells

The virus or particle that has been incorporated by the chimeric viral surface polypeptide is then administered to the animal for exposure to target cells. The target cells of the invention express receptors of the LDL-receptor family, including but not limited to LDLR, VLDLR-1, VLDLR-2, LRP, and gp330. Although the invention is not limited to any theories of how the invention works, a particular advantage of this invention over the prior art is that ligands that bind receptors of the LDL-receptor family are internalized after binding their receptors. Where the ligand is an integral part of the above described chimera, the virus or particle incorporated by the chimera is internalized with its ligand portion after binding a receptor.

A particular example where the binding pair of the ligand and the receptor accomplishes the internalization of the ligand in those cells expressing the receptor is the example of the PAI-1 ligand, and PAI-1 ligand complexes. Although the invention is not limited to theories of mechanism, it is believed that PAI-1 binds uPAR bound uPA on the cell surface and then the newly formed complex of PAI-1:uPA binds LRP, also on the cell surface. PAI-1 forms a covalent bond with uPA which accounts in part for the efficient internalization of this ligand complex. A virus or particle is internalized when the ligand portion of the chimeric viral surface protein comprising, for example, the portion of PAI-1

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sufficient for binding uPAR bound uPA and the portion also sufficient for then binding LRP, binds uPA and then binds LRP.

Although the invention is not limited to any theories of mechanism, the internalization of ligands via the LRP pathway may bring the additional advantage that processing of the viral or particle contents occurs under lysosomal conditions including
5 acidic pH which may facilitate efficient release of the viral or particle contents into the cell.

Example 5

Altered Particles

10 The invention also includes an altered particle for cell-specific delivery. The altered particle is constructed by attaching a portion of a ligand specific for a receptor of the LDL-receptor family to a particle and using the ligand to target cells for internalization of the particle and its molecular contents. Altered particles contain molecular contents that have a ligand portion attached to the particle. The ligand portion
15 is derived from the ligands of receptors of the LDL-receptor family, including those described herein. The ligand portion retains at least the portion specific for binding the receptor, and sufficient portion for attaching to the particle. If the ligand portion forms a ligand complex, the ligand portion retains in addition a portion capable of binding to the polypeptide or polypeptides of that form the ligand complex. The internalization of the
20 ligand-attached altered particle occurs by binding of the ligand portion to the receptor on the cell surface. Where, for example, the PAI-1 ligand is used, covalent binding occurs between the ligand portion comprising PAI-1 and uPAR bound uPA. The ligand complex, also still attached to the particle, then binds the cell surface receptor LRP and is internalized in the cell. The ligand or ligand portion is attached to the particle by any
25 means known in the art for such manipulation, including chemical crosslinking, photopolymerization, UV cross-linking, and other techniques known in the art.

The molecular contents of the viruses and particles include, but are not limited to, coding sequences, and their regulatory sequences and vectors, for expression of polypeptides in the target cells. The molecular contents of the particles also includes

polynucleotides such as antisense oligonucleotides and ribozymes for delivery to the cell. The contents also include any number of therapeutics, including polypeptides or proteins, drugs, small molecules including peptides and peptoids, and combinations of all of these. Where an altered particle is concerned, the altered particle can be filled with molecular
5 contents before or after the alteration of the particle by attaching a ligand portion.

Example 6

Polypeptides Expressable in the Target Cells

The polypeptides expressable in a target cell by the invention can be any polypeptide, including, for example, the coding sequence from any protein or polypeptide
10 or part of a protein or polypeptide having biological activity including, but not limited to, for example, a cytokine, a protein hormone, an enzyme, a protease, a protease inhibitor, a growth factor, a differentiation factor, cell surface receptor, or an antibody or antigen.

For delivery of coding sequences using viral vectors, any of a number of viral
15 vectors can be used, as described in Jolly, *Cancer Gene Therapy* : 51-64 (1994). For example, the coding sequence can be inserted into plasmids designed for expression in retroviral vectors, as described in Kimura *et al.*, *uman Gene Therapy* (1994) : 845-852, adenoviral vectors, as described in Connelly *et al.*, *uman Gene Therapy* (1995) : 185-193, adeno-associated viral vectors, as described in Kaplitt *et al.*, *Nature Genetics*
20 (1994) : 148-153 and sindbis vectors. Promoters that are suitable for use with these vectors include, for example, the Moloney retroviral LTR, CMV promoter and the mouse albumin promoter. Replication incompetent free virus can be produced and injected directly into the animal or human or by transduction of an autologous cell *in vivo*, followed by injection *in vivo* as described in Zatloukal *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) : 5148-5152.
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Expression of the coding sequence *in vivo* upon delivery for gene therapy purposes by viral vectors can be regulated for maximal efficacy and safety by use of regulated gene expression promoters as described in Gossen *et al.*, *Proc. Natl. Acad. Sci. USA* (1992) : 5547-5551. For example, the coding sequence can be regulated by

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tetracycline responsive promoters. These promoters can be regulated in a positive or negative fashion by treatment with the regulator molecule. Other molecular contents for delivery to target cells can include in addition, other therapeutic agents, for example, proteins, polypeptides, non-protein therapeutics, ribozymes, antisense oligonucleotides, peptides, peptoids, other small molecules, and drugs.

Example 7

Administration of the Particles Incorporated by the Chimeras

A particle incorporated by a chimera of the invention, or the altered particle, are administered by any means known for the delivery of particles or viruses to an animal or human, including delivery locally, intravenously, by particle gun, pump, catheter, cannulization, inhalation or other means known in the art. The virus or particle may be administered in a pharmaceutically acceptable composition including but not limited to Depofoam™ and Focalgel™.

Once in the animal, the virus, particle or altered particle is internalized by cells that express the receptor of the LDL-receptor family with which the ligand forms a binding pair. Once the virus or particle is internalized in a cell depending on the molecular contents contained in the virus or particle, a coding sequence is expressed, or the contents are released into the cell.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

What is claimed:

1. A chimeric polynucleotide encoding a chimera of a ligand and a viral surface protein for use in delivery of a virus or a particle containing molecular contents for internalization into target cells comprising,
a first polynucleotide sequence encoding all or a portion of a ligand of a receptor from the LDL-receptor family, and
a second polynucleotide sequence encoding all or a portion of a viral surface protein, wherein
the expression product of the chimeric polynucleotide forms a chimeric viral surface polypeptide capable of forming an incorporation aggregate for incorporation of a virus or particle, and capable of forming a binding pair with the receptor for internalization of the virus or particle incorporated by the chimeric viral surface polypeptide into a target cell expressing the receptor, and wherein the virus or particle comprises molecular contents for delivery to a target cell.
2. The chimeric polynucleotide of claim 1 wherein the ligand forms a covalent bond with uPAR bound uPA forming a ligand:uPA complex, wherein the ligand:uPA complex binds LRP and is internalized in a target cell.
3. The chimeric polynucleotide of claim 1 wherein the chimeric viral surface polypeptide comprises a viral surface protein of a virus comprising a lipid bilayer membrane.
4. The chimeric polynucleotide of claim 3 wherein the virus is a retrovirus.
5. The chimeric polynucleotide of claim 1 wherein the chimeric viral surface polypeptide comprises a capsid protein from a virus.
6. The chimeric polynucleotide of claim 5 wherein the ligand forms a covalent bond with uPAR bound uPA forming a ligand:uPA complex, wherein the ligand:uPA complex binds LRP and is internalized in a target cell.
7. The chimeric polynucleotide of claim 1 wherein the molecular contents comprise a polynucleotide encoding a polypeptide for expression in the target cell.

8. The polynucleotide of claim 7 also comprising a vector selected from the group consisting of a viral vector, a retroviral vector, or a non-viral vector.
9. The chimeric polynucleotide of claim 1 wherein the molecular contents are selected from the group consisting of a protein, a small molecule, a ribozyme, and an antisense oligonucleotide.
10. The chimeric polynucleotide of claim 1 wherein the receptor from the LDL-receptor family is selected from the group consisting of LDLR, VLDLR-1, VLDLR-2, LRP, and gp330.
11. The chimeric polynucleotide of claim 10 wherein the target cell also expresses uPAR.
12. The chimeric polynucleotide of claim 11 wherein the ligand forms a covalent bond with uPAR bound uPA forming a ligand:uPA complex, wherein the ligand:uPA complex binds LRP and is internalized in a target cell.
13. The chimeric polynucleotide of claim 1 wherein the ligand is a serine protease inhibitor (SERPIN).
14. The chimeric polynucleotide of claim 13 wherein the SERPIN is selected from the group consisting of PAI-1, protease nexin 1, α -1-antitrypsin, and α -1-antichymotrypsin.
15. The chimeric polynucleotide of claim 13 wherein the SERPIN forms a ligand complex, and the ligand complex binds a receptor from the LDL-receptor family.
16. The chimeric polynucleotide of claim 1 wherein the ligand is tissue factor pathway inhibitor (TFPI).
17. The chimeric polynucleotide of claim 1 wherein the viral surface protein is derived from a viral surface protein selected from the group consisting of an adeno-virus, an adeno-associated virus, a herpes simplex virus, a cytomegalovirus, a retrovirus, an influenza virus, a sindbis virus, semliki forest virus and a vesicular stomatitis virus.
18. The chimeric polynucleotide of claim 1 wherein the molecular contents comprise a viral genome and the viral genome is selected from the group consisting of an adeno-virus, an adeno-associated virus, a retrovirus, a sindbis virus, and a BK virus.

19. The chimeric polynucleotide of claim 1 wherein the target cell is a eukaryotic cell.
20. The chimeric polynucleotide of claim 19 wherein the eukaryotic cell is a mammalian cell.
21. The chimeric polynucleotide of claim 20 wherein the mammalian cell is a human cell.
22. A chimeric polypeptide encoded by the chimeric polynucleotide of claim 1.
23. The chimeric polynucleotide of claim 7 wherein the polynucleotide encoding a polypeptide for expression in the target cell is selected from the group of biologically active proteins consisting of cytokines, protein hormones, enzymes, proteases, growth factors, differentiation factors, transcription factors and receptors.
24. The chimeric polynucleotide of claim 1 wherein the particle or altered particle is a particle or vesicle selected from the group consisting of a liposome, a cyclodextrin liposome, and a heterovesicular liposome.
25. A chimeric polypeptide for incorporation of a virus or particle for targeting a cell for internalization of the virus or particle for delivery of viral or particle contents comprising,
 - a first polypeptide sequence including all or a portion of a ligand of a receptor from the LDL-receptor family, and
 - a second polypeptide sequence including all or a portion of a viral surface protein, wherein
 - the chimeric polypeptide comprises an incorporation aggregate for incorporation of a virus or particle to form an incorporated virus or incorporated particle for targeting a cell expressing the receptor, wherein
 - the ligand forms a binding pair with the receptor for internalization of the incorporated virus or incorporated particle and wherein
 - the virus or particle comprises molecular contents for delivery to the target cell.

26. The chimeric polypeptide of claim 25 wherein the chimeric viral surface protein comprises a viral surface protein from a virus comprising a lipid bilayer membrane.
27. The chimeric polypeptide of claim 26 wherein the viral surface protein is from a retrovirus.
28. The chimeric polypeptide of claim 25 wherein the viral surface protein comprises a capsid protein from a virus.
29. The chimeric polypeptide of claim 25 wherein the ligand forms a covalent bond with uPAR bound uPA forming a ligand:uPA complex, wherein the ligand:uPA complex binds LRP and is internalized in a target cell.
30. The chimeric polypeptide of claim 25 where in the ligand is a serine protease inhibitor (SERPIN).
31. The chimeric polypeptide of claim 30 wherein the SERPIN is selected from the group consisting of PAI-1, protease nexin 1, α -1-antitrypsin, and α -1-antichymotrypsin.
32. The chimeric polypeptide of claim 25 wherein the ligand is TFPI.
33. The chimeric polypeptide of claim 25 wherein the receptor is selected from the group consisting of LDLR, VLDLR-1, VLDLR-2, LRP, and gp330.
34. The chimeric polypeptide of claim 33 wherein the target cell also expresses uPAR.
35. The chimeric polypeptide of claim 34 wherein the ligand forms a covalent bond with uPAR bound uPA forming a ligand:uPA complex, wherein the ligand:uPA complex binds LRP and is internalized in a target cell.
36. The chimeric polypeptide of claim 35 wherein the ligand is PAI-1.
37. The chimeric polynucleotide of claim 25 wherein the viral surface protein is derived from one selected from the group consisting of surface proteins of an adeno-virus, an adeno-associated virus, a herpes simplex virus, a cytomegalovirus, a retrovirus, an influenza virus, a sindbis virus, semliki forest virus and a vesicular stomatitis virus.

38. The chimeric polypeptide of claim 25 wherein the contents comprise a polynucleotide encoding a polypeptide for expression in the target cell.
39. The polynucleotide of claim 38 also comprising a vector wherein the vector is a viral vector, a retroviral vector, or a non-viral vector.
40. The chimeric polypeptide of claim 25 wherein the contents comprise one from the group of a protein, a ribozyme, a small molecule inhibitor and an anti-sense oligonucleotide.
41. The chimeric polypeptide of claim 25 wherein molecular contents comprise a viral genome and the viral genome is selected from the group consisting of an adeno-virus, an adeno-associated virus, a retrovirus, a sindbis virus, and a BK virus.
42. The chimeric polypeptide of claim 25 wherein the target cell is a eukaryotic cell.
43. The chimeric polypeptide of claim 42 wherein the eukaryotic cell is a mammalian cell.
44. The chimeric polypeptide of claim 43 wherein the mammalian cell is a human cell.
45. The chimeric polynucleotide of claim 25 wherein the particle is selected from the group consisting of a liposome, a cyclodextrin liposome, and a heterovesicular liposome.
46. A method of targeting and internalizing a virus or particle comprising the steps of (a) constructing the chimeric polynucleotide of claim 1, (b) expressing the chimeric polynucleotide to form a chimeric viral surface polypeptide (c) incorporating the chimeric viral surface polypeptide into the surface of a virus or particle containing molecular contents to form an incorporated virus or particle, (d) bringing the incorporated virus or particle in contact with a target cell expressing the receptor (e) internalizing the virus or particle into the target cell by binding of the ligand with the receptor.

47. The method of claim 46 wherein the step of expressing the chimeric polynucleotide occurs in a cell that contains the virus.
48. The method of claim 46 wherein the receptor is selected from the group consisting of LDLR, VLDLR-1, VLDLR-2, LRP, and gp330.
49. The method of claim 48 wherein the ligand forms a covalent bond with uPAR bound uPA forming a ligand:uPA complex, wherein the ligand:uPA complex binds LRP and is internalized in a target cell.
50. The method of claim 46 wherein the ligand is a SERPIN.
51. The method of claim 50 wherein the SERPIN is selected from the group consisting of PAI-1, protease nexin 1, α -1-antitrypsin, and α -1-antichymotrypsin.
52. The method of claim 46 wherein the ligand is TFPI.
53. The method of claim 46 wherein the viral surface protein is derived from a surface protein from one selected from the group consisting of an adeno-virus, an adeno-associated virus, a herpes simplex virus, a cytomegalovirus, a retrovirus, an influenza virus, a sindbis virus, a semliki forest virus and a vesicular stomatitis virus.
54. The method of claim 46 wherein the virus comprises a viral genome and the viral genome is selected from the group consisting of an adeno-virus, an adeno-associated virus, a retrovirus, a sindbis virus, and a BK virus.
55. The method of claim 46 wherein the molecular contents comprise a polynucleotide encoding a polypeptide for expression in a target cell.
56. The method of claim 55 wherein the polynucleotide encoding a polypeptide for expression in a target cell is selected from the group of biologically active proteins consisting of cytokines, protein hormones, enzymes, proteases, growth factors, differentiation factors, transcription factors, and receptors.
57. The method of claim 46 wherein the molecular contents comprise one selected from the group consisting of a protein, a ribozyme, and an anti-sense oligonucleotide.
58. The method of claim 46 wherein the particle is selected from the group consisting of a liposome, a cyclodextrin liposome, and a heterovesicular liposome.

59. An altered particle designed for delivery to target cells for internalization of molecular contents contained in the particle comprising
- a particle with the ability to contain molecular contents
 - a polypeptide moiety comprising all or a portion of a ligand of a receptor from the LDL-receptor family, wherein
 - the particle and the ligand portion are connected to one another to form an altered particle for delivery of the molecular contents of the particle to a target cell.
60. The altered particle of claim 59 wherein the molecular contents comprise a polynucleotide encoding a polypeptide for expression in the target cell.
61. The particle of claim 60 also comprising a vector wherein the vector is selected from one from the group consisting of a viral vector, a retroviral vector, or a non-viral vector
62. The altered particle of claim 59 wherein the molecular contents comprise one selected from the group consisting of a protein, a small molecule, a ribozyme, and an antisense oligonucleotide.
63. The altered particle of claim 59 wherein the receptor from the LDL-receptor family is selected from the group consisting of LDLR, VLDLR-1, VLDLR-2, LRP, and gp330.
64. The altered particle of claim 63 wherein the ligand forms a covalent bond with uPAR bound uPA forming a ligand:uPA complex, wherein the ligand:uPA complex binds LRP and is internalized in a target cell.
65. The altered particle of claim 64 wherein the ligand is PAI-1.
66. The altered particle of claim 59 wherein the ligand is a serine protease inhibitor (SERPIN).
67. The altered particle of claim 66 wherein the SERPIN is selected from the group consisting of PAI-1, protease nexin 1, α -1-antitrypsin, and α -1-antichymotrypsin.
68. The altered particle of claim 59 wherein the ligand is tissue factor pathway inhibitor (TFPI).
69. The altered particle of claim 59 wherein the target cell is a eukaryotic cell.

70. The altered particle of claim 69 wherein the eukaryotic cell is a mammalian cell.

71. The altered particle of claim 70 wherein the mammalian cell is a human cell.

72. The altered particle of claim 60 wherein the polynucleotide encoding a polypeptide for expression in the target cell is one selected from the group of biologically active proteins consisting of cytokines, protein hormones, enzymes, proteases, growth factors, differentiation factors, transcription factors and receptors.

73. The altered particle of claim 59 wherein the particle is selected from the group consisting of a liposome, a cyclodextrin liposome, and a heterovesicular liposome.

INTERNATIONAL SEARCH REPORT

Int: onal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 C12N15/62 C12N15/86 A61K48/00 C07K14/81
C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93 24141 A (UNIV TOLEDO) 9 December 1993 see the whole document ---	1-15, 17-31, 33-51, 53-67, 69-73
Y	WO 93 20221 A (YOUNG ALEXANDER T) 14 October 1993 see the whole document --- -/--	1-4, 6-15, 17-27, 29-31, 33-51, 53-67, 69-73

☒ Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93 25234 A (UNIV CALIFORNIA) 23 December 1993 see the whole document ---	1-15, 17-31, 33-51, 53-67, 69-73
Y	WO 94 27643 A (TARGETED GENETICS CORP ;PAUL RALPH W (US); OVERELL ROBERT (US)) 8 December 1994 see the whole document ---	1-4,6-8, 10-15, 17-27, 29-31, 33-39, 41-51, 53-56, 58-61, 63-67, 69-73
A	THE EMBO JOURNAL, vol. 9, no. 4, 1990, pages 1079-1085, XP002027849 CUBELLIS, M.V., ET AL . : "RECEPTOR-MEDIATED INTERNALIZATION AND DEGRADATION OF UROKINASE IS CAUSED BY ITS SPECIFIC INHIBITOR PAI-1" see the whole document ---	1-73
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 22, June 1994, pages 15827-15832, XP002027850 WILLNOW, T.E., ET AL . : "MOLECULAR DISSECTION OF LIGAND BINDING SITES ON THE LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN" see the whole document -----	1-73

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l Application No

PCT/US 96/19196

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9324141 A	09-12-93	AU 4394293 A EP 0643584 A	30-12-93 22-03-95
WO 9320221 A	14-10-93	AU 3940293 A CA 2133411 A EP 0633943 A JP 7505773 T	08-11-93 14-10-93 18-01-95 29-06-95
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WO 9427643 A	08-12-94	AU 7097494 A	20-12-94

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